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# MONOCLONAL ANTIBODY TO YopE FACILITATES DETECTION AND STUDY OF PLASMID-BEARING *YERSINIA* SPP.

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## Summary

Monoclonal antibody 3.2C was specific for YopE protein and could be used as a marker for all P+ *Yersinia* serotypes tested. Western blotting was used to detect YopE in cell lysates, on the surface of cells and as a released protein in the medium following a temperature shift from 25° to 37° in growth medium containing low levels of calcium (<350 μM). The utility of the MAb was demonstrated in experiments that further defined optimal conditions for synthesis and detection of cell-associated and released YopE.

## Introduction

Pathogenic *Yersinia* spp. cause diseases in humans and animals (3) ranging from mild gastrointestinal disorders (*Y. enterocolitica, Y. pseudotuberculosis*) to severe involvement of the lymphatic system, bacteremia, organ lesions and death (*Y. pestis*). Serotypes of *Y. enterocolitica* are the most prevalent *Yersinia* spp. isolated from animal and human infections (3). Virulence factors are complex, but all pathogenic serotypes harbor a virulence plasmid of about 70 kb. A number of plasmid genes are regulated by a low-calcium virulence regulon (Lcr) and are expressed following a temperature

shift from 25° to 37° under conditions similar to those experienced by the organisms as they move from natural environments into the interior of phagolysosomes of macrophages (see ref. 11). We have generated monoclonal antibodies (MAb) to one of these proteins (YopE) using the 25 kd YopE protein isolated from *Y. enterocolitica* serotype 0:8 as the immunizing antigen. In addition to being a general marker for the presence of the virulence plasmid, YopE deserves additional study for its role in pathogenesis. It has been implicated as an important cell-surface anti-phagocytic factor (9) and in the disruption of actin microfilaments in mammalian cells (10). Mutations in YopE result in loss of cytotoxicity for cells in tissue culture (9, 10). Yop proteins also have been detected in synovial fluids of patients suffering from *Yersinia*-triggered arthritis (6).

## Materials and Methods

*Bacterial Strains.* Clinical isolates of *Y. enterocolitica* serotype O:3 (CI) and O:9 (CI) were obtained from the Centers for Disease Control, Atlanta, GA. *Y. enterocolitica* serotypes O:3 (E739), O:5,27 (E614), O:8 (E663), O:21 (E780) and O:4,32 (E701) were provided by Dr. D. A. Schiemann (Department of Microbiology, Montana State University), and *Y. pseudotuberculosis* (PA1) and *Y. pseudotuberculosis* (YPIII) by Dr. R. R. Brubaker (Department of Microbiology and Public Health, Michigan State University). *Y. enterocolitica* serotype O:8 (E663) was the primary organism used in this study. Isogenic plasmid-bearing ($P^+$) and plasmidless ($P^-$) cells were isolated as described by Mazigh *et al.* (8). All strains were screened for the presence of plasmid as described previously (2).

*Growth Conditions.* $P^+$ and $P^-$ cells of *Y. enterocolitica* serotype O:8 (E663) were grown at 25° or 37° in Trypticase Soy Broth (TSB), Trypticase Soy Agar (TSA), Brain Heart Infusion (BHI) (Difco Laboratories, Detroit, MI), or trypticase soy agarose (TSO), prepared as described (1). For experiments shown in Figures 2 and 3, the organism was grown in BHI at 25°C overnight, and the optical density of the culture was adjusted to 0.4 at 600 nm ($A_{600}$). The bacterial culture was diluted 1:20 with fresh BHI, the temperature shifted from 25° to 37°, and incubation continued with rotatory shaking at 200 rpm for the duration of the experiment. Bacteria were harvested by centrifugation at 10,500 X *g* for 20 min at 4°, washed once with PBS (0.01 M phosphate in 0.14 M

NaCl, pH 7.2), adjusted to $A_{600}$ of 0.5, and sonicated to prepare a cell lysate. Released proteins were recovered from the culture supernatant by ammonium sulfate precipitation (5).

*SDS-PAGE, ELISA, and Western Blotting.* Sonicated and ammonium sulfate precipitated protein samples were solubilized in SDS-PAGE sample buffer (10% SDS, 10% 2-mercaptoethanol, 50% sucrose in 0.125 M Tris-HCl, pH 6.8). Procedures for electrophoresis with 12% separating gels, Western blotting, and staining with Commassie blue were the same as previously described (7).

*Production of Monoclonal Antibodies.* Purified YopE protein, isolated by preparative SDS-PAGE, was used as the immunogen to generate MAbs. Procedures for immunization of animals, cell fusion, development of hybridomas, and isolation and characterization of MAbs were as previously described (7). After cell fusion, 360 hybridomas were obtained and screened for antigen-specific antibodies. Based on ELISA titers and patterns of cross-reactivities, MAbs 3.2C and 23.2C were selected and studied in detail. The isotypes of the MAbs were IgG2a with κ light chain and IgG2b with κ light chain, respectively (7).

## Results and Discussion

Western blotting demonstrated that MAbs 3.2C or 23.2C could detect YopE in total cell lysates, in cell washes and in proteins released into culture supernatants (Fig. 1). The protein was not expressed at 25°, was expressed at 37° (Fig, 1A), and at intermediate $Ca^{2+}$ concentration (TSO; 311 μM; ref. 1), and was common to all $P^+$ strains of *Yersinia* tested (Fig. 1C). MAb 3.2C reacted only with YopE without cross-reacting with any other cellular antigens or with proteins from an extensive panel of other gram- negative organisms (not shown), while MAb 23.2C cross-reacted with a 36 KDa protein that was not excreted and was not under temperature or $Ca^{2+}$ regulation (compare Fig. 1A and 1B). Heesemann *et al.* (4) and Weninger *et al.* (12) also have produced MAbs against YopE, but some of their MAbs were able to distinguish between the protein from *Y. enterocolitica* and *Y. pseudotuberculosis* . As shown in Figure 1A, appreciable amounts of YopE were produced when *Y. enterocolitica* serotype O:8 was grown on an agar-based medium containing high calcium concentrations (TSA; 1,400 μM), although it is likely that much of the calcium in

agar media is in a complexed rather than free form. Bhaduri (1) also has reported that plasmid-mediated proteins were expressed by *Y. enterocolitica* serotypes grown on high and low calcium media at 37°.

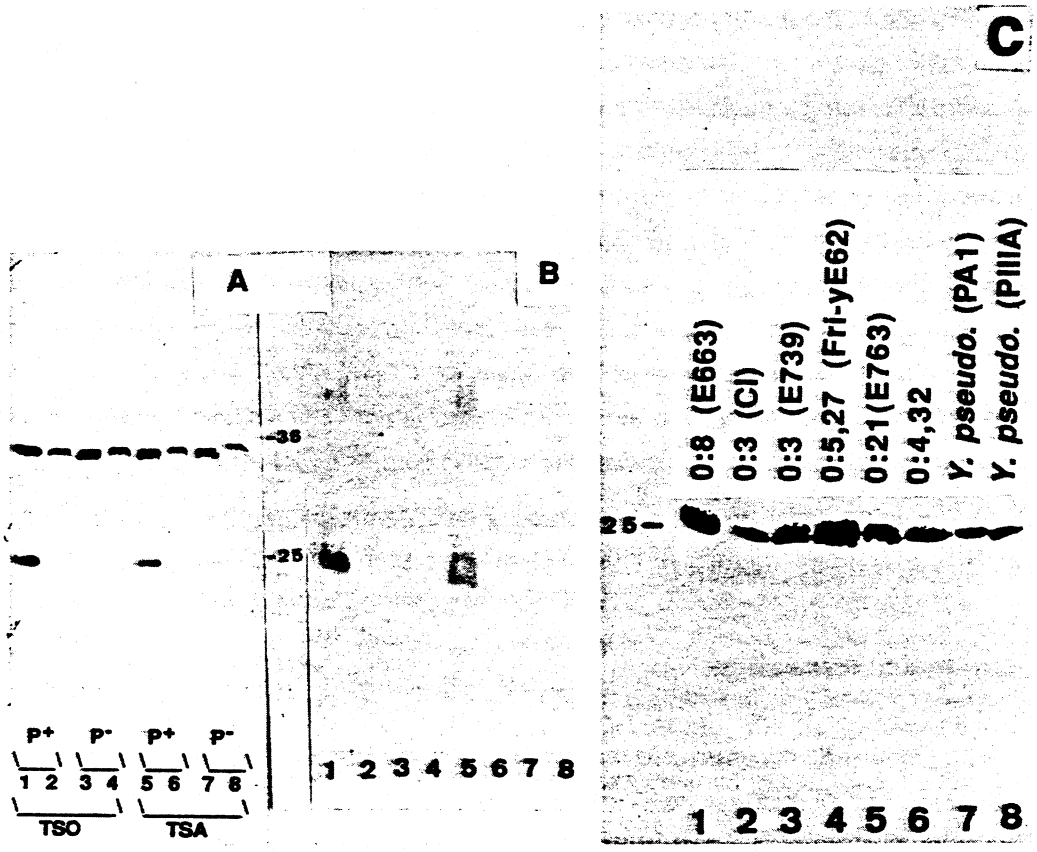


**FIG. 1.** Western blotting of (A) cell lysates, (B) cell washes and (C) proteins released into culture supernatants. For (A) and (B), $P^+$ and $P^-$ cells of *Y. enterocolitica* serotype O:8 (E663) were grown overnight on TSO or TSA. The cells were collected and washed with PBS and a cell lysate prepared by sonication. Cell surface proteins were washed from cells (5) and collected by ammonium sulfate precipitation. Proteins were separated by SDS-PAGE, and gels were probed with MAb 23.2C diluted 1:4000. The MAb detected YopE (25kd) and a 36 kd protein that was not regulated by temperature or $Ca^{2+}$. Each lane contained 20 μg protein in (A) and 10 μg in (B). Lanes 1, 3, 5, and 7 from cells grown at 37°, lanes 2, 4, 6, and 8 from cells grown at 25°. (C) Different strains of Yersinia were grown at 37° in TSB overnight, and proteins released into the medium were collected by ammonium sulfate precipitation. Separated proteins (10 μg / lane) were probed with MAb 3.2C which is specific for YopE. Serotype numbers refer to *Y. enterocolitica*. *Y. pseudotuberculosis* strains were grown in the presence of 10 mM ethylene glycol-*bis*(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA).

Large amounts of YopE were expressed in the late exponential growth phase when *Y. enterocolitica* was grown in broth medium such as TSB and BHI without need for depletion of calcium by the use of calcium chelators as is commonly recommended (see ref. 1, 2). However, only a small amount of the protein was produced in $P^+$ cells of *Y. pseudotuberculosis* in TSB (not shown) unless the medium was supplemented with 10 mM EGTA (Fig. 1C, lanes 7 and 8).

The time course for synthesis and release of YopE by serotype O:8 (E663) in BHI after a temperature shift to 37° was examined along with the effects of inhibitory levels of calcium added at various times of incubation. As shown in Figure 2, protein release increased over the 5 h incubation and correlated with increased cell growth. YopE was readily detected at 3 h. At the end of the 5 h incubation period, the $A_{600}$ of the cultures was 0.70 for the organism grown in BHI and 1.03 for the organism grown in BHI supplemented with 1.0 mM calcium chloride at 1 h, illustrating the stimulatory effect of calcium on cell growth. In comparison, when calcium was added at 2 or 3 h, appreciable amounts of the released proteins accumulated by 5 h (lanes 4 and 6), indicating that their release continued for about 2 h after the addition of calcium. A common problem in identifying pathogenic isolates from natural sources or clinical samples is due to their conversion from $P^+$ to $P^-$ during isolation and growth, particularly at 37° (1, 2). Our data indicate that YopE is readily detected in organisms grown to an $A_{600}$ of about 0.3 during a relatively brief incubation in BHI at 37°; conditions that should minimize loss of plasmid.

MAb 3.2C was used to examine more closely the effects of calcium concentrations between 245 and 1,245 μM on synthesis of Yop proteins by cells growing in BHI. Increased calcium concentration in the growth medium led to inhibition of intracellular accumulation of the 25 KDa protein as shown by Western blotting of cell lysates as shown in Figure 3B, lanes 1, 3, 5, and 7. YopE was not readily seen in Commassie blue stained gels of cell lysates (Fig. 3 A). From Figure 3B (lanes 2, 4, 6, and 8), it is clear that the optimal concentration of calcium in liquid growth medium for secretion of YopE was about 345 μM (BHI; 245 μM, plus a 0.1 mM calcium chloride supplement). Decreased expression of YopE and of all released proteins was found at higher calcium concentrations, and YopE was not detectable at all by Western blotting if the growth medium was supplemented with 1.0 mM or more calcium chloride.

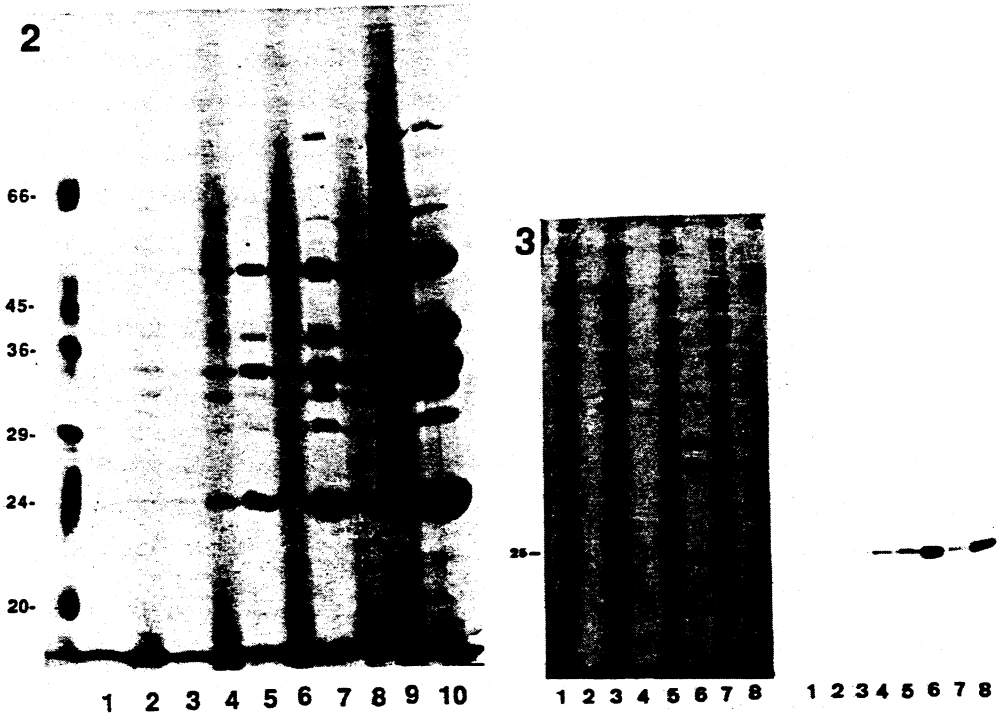


**FIG. 2.** Effect of exogenous calcium on expression of released proteins during growth of *Y. enterocolitica* serotype O:8 in BHI following a temperature shift from 25° to 37°. SDS-PAGE gels were stained with Commassie blue. Lanes 1, 3, 5, 7, and 10 show proteins released into the medium at 1, 2, 3, 4, and 5 h, respectively. Lanes 2, 4, 6, 8, and 9 show proteins released from the organism by five h when 1.0 mM calcium chloride was added at 1, 2, 3, 4, or 5 h, respectively.

**FIG. 3.** Effect of calcium concentration on expression of YopE and other plasmid-encoded proteins. Cell homogenates (30 µg/lane) and released proteins (from 2 ml supernatant/lane) were prepared from cultures of *Y. enterocolitica* serotype O:8 which were grown at 37° for 4 h. The separated proteins were (A) stained with Commassie blue or (B) blotted with MAb 3.2C. Lanes 1, 3, 5, and 7, cell homogenates from the organism grown in BHI supplemented with 1.0, 0.5, 0.1, or 0 mM calcium chloride, respectively. Lanes 2, 4, 6, and 8, proteins from the culture supernatants from cultures supplemented with 1.0, 0.5, 0.1 or 0 mM calcium chloride, respectively.

YopE was also secreted into the medium by a variety of *Yersinia* strains grown in overnight stationary cultures in TSB (Fig. 1C). Thus, we conclude that media with

defined, intermediate levels of calcium were optimal for expression of plasmid-encoded proteins of $P^+$ *Y. enterocolitica* serotype O:8 (E663). Calcium depletion or chelation of calcium with EGTA were not necessary. In fact, we observed inhibition of released proteins of 38 and 43 kd during exponential growth on BHI supplemented with 10 mM EGTA (not shown). Apparently, chelating agents such as oxalate and EGTA may be toxic or bind other essential minerals in the growth medium (1). It must be emphasized that conditions for optimum expression of released proteins of *Yersinia* spp. cannot be generalized. Low-calcium medium was indeed required for optimum expression of released proteins of the *Y. pseudotuberculosis* strains studied (see Fig.1C).

These experiments demonstrate that MAbs specific for YopE are useful for rapid differentiation between $P^+$ and $P^-$ strains of Yersinia under conditions designed to minimize loss of plasmids, and for further studies on the synthesis and secretion of the protein itself. They also could be used as an analytical tool for studying the role of released protein in the pathogenic manifestations of the organism.

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## References

1. Bhaduri S. Calcium responsive expression of plasmid-mediated outer membrane proteins from *Yersinia enterocolitica* grown on solid media. J Industr Microbiol 1990; **5:** 207-14.
2. Bhaduri S, Jones CT, Taylor MM, Lachica RV. Simple assay of calcium dependency for virulent plasmid-bearing clones of *Yersinia enterocolitica*. J Clin Microbiol 1990; **28:** 798-800.
3. Cornelis G, Laroche Y, Balligand G, Sorry M-P, Wauters G. *Y. enterocolitica*, a primary model for bacterial invasiveness. Rev Infect Dis 1987; **9**: 64-87.
4. Heesemann J, Kalthoff H, Koch F. Monoclonal antibodies directed against plasmid-encoded released proteins of enteropathogenic *Yersinia*. FEMB Microbiol Letters 1986; **36:** 15-9.
5. Heesemann J, Gross U, Schmidt N, Laufs R. Immunological analysis of plasmid-encoded proteins released from enteropathogenic *Yersinia* sp. grown in calcium-deficient media. Infect Immun 1986; **54:** 561-7.

6. Lahesmaa-Rantala R, Heesemann J, Lehtonen O-P, Granfors K, Toivanen A. Avidity of antibodies against released proteins of *Yersinia* spp.: comparison of patients with or without reactive arthritis. Ann Rheum Dis 1989; **48:** 1003-6.
7. Li Q, DiSanto ME, Magee WE. A rapid method for detection of *Yersinia enterocolitica* serotype 0:3 in pig feces using monoclonal antibodies. Vet Microbiol 1992; **32:** 29-41.
8. Mazigh D, Alonso JM, Mollaret HH. Simple method for demonstration of differential colony morphology of plasmid-associated virulent clones of *Yersinia enterocolitica*. J Clin Microbiol 1983; **17:** 555-7.
9. Rosqvist R, Forsberg A, Rimpilainen M, Bergman T, Wolf-Watz H. The cytotoxic protein YopE of *Yersinia* obstructs the primary host defence. Mol Microbiol 1990; **4:** 657-67.
10. Rosqvist R, Forsberg A, Wolf-Watz H. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infect Immun 1991; **59:** 4562-9.
11. Straley SC. The low-$Ca^{2+}$ response virulence regulon of human-pathogenic yersiniae. Microb Path 1991; **10:** 87-91.
12. Weninger J, Schoerner C, Wartenberg K, Rollinghoff M. Detection of cross-reacting epitopes on plasmid-encoded outer membrane proteins of enteropathogenic *Yersinia* by monoclonal antibodies. Med Microbiol Immunol 1989; **178:** 45-51.